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Thomas Lacoue-Labarthe, Sophie Martin, François Oberhänsli, Jean-Louis Teyssié, Ross Jeffree, Jean-Pierre Gattuso, Paco Bustamante

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T. Lacoue-Labarthe[1,2,3,*], S. Martin[2,†], F. Oberhänsli[2], J.-L. Teyssié[2], R. Jeffree[2], J.-P. Gattuso[3,4], P. Bustamante[1]

[1]Littoral Environnement et Sociétés (LIENSs), UMR 6250 CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, France

[2]International Atomic Energy Agency - Environment Laboratories, 4 Quai Antoine Ier, Monaco

[3]INSU-CNRS, Laboratoire d'Océanographie de Villefranche, B.P. 28, 06234 Villefranche-sur-mer Cedex, France

[4]Université Pierre et Marie Curie, Observatoire Océanologique de Villefranche, 06230 Villefranche-sur-mer, France

* Corresponding author: Dr. Thomas Lacoue-Labarthe
IAEA – Environmental Laboratories
4 Quai Antoine Ier
MC 98 000 Monaco
Monaco

Phone: +377 97 97 72 54
E-mail: tlacouel@gmail.com

[†] present address: CNRS, Laboratoire Adaptation et Diversité en Milieu Marin, Station Biologique de Roscoff, Place Georges Teissier, 29682 Roscoff Cedex, France, sophie.martin@sb-roscoff.fr

Abstract. The increasing release of CO_2 by human activities leads to ocean acidification and global warming. Both those consequences (*i.e.,* increase in seawater pCO_2 and temperature) may drastically affect physiology of marine organisms. The effects of low pH and elevated temperature on the bioaccumulation of radionuclides (^{241}Am, ^{134}Cs) and trace elements (^{60}Co, ^{54}Mn or ^{75}Se) were studied during the embryonic development of the common cuttlefish *Sepia officinalis*. The lowered accumulation of essential ^{60}Co and ^{54}Mn with decreasing pH was larger at 16°C than at 19°C. Se was not detected in the embryo whereas it penetrated through the eggshell, suggesting that an alternative pathway ensures the supply of this essential metal for the embryo. ^{241}Am was totally retained by the eggshell irrespective of pH and temperature. Finally, the amount of Cs found in the peri-vitelline fluid increased with decreasing pH likely because of an enhanced swelling of the cuttlefish egg under elevated CO_2.

Keywords: cephalopod, ocean acidification, ocean warming, eggshell, embryonic development, metal.

INTRODUCTION

Climate change due to the increasing atmospheric carbon dioxide (CO_2) concentrations in the atmosphere is now considered to be a major threat to biodiversity as well as to the structure and function of ecosystems (Mc Carthy, 2001). Although all the causes are still subjected to debate (Sharp, 2003), it is widely accepted that human activities such as fossil fuel combustion, cement production and change in land use (Hansen et al., 2007) drove the rising global atmospheric concentration of CO_2 from a pre-industrial value of approximately 280 parts per million (ppm) to 384 ppm in 2007 (~387 ppm in 2009) (Anonymous, 2010). Moreover, models project that the CO_2 concentration will double by midcentury (> 500 ppm) and could reach between 730 and 1020 ppm in 2100 (IPCC, 2007). The accumulation of greenhouse gases will have profound consequences among which the global warming. Temperature is expected to increase on average by 3°C at the Earth's surface over the course of this century (IPCC, 2007). Furthermore, the precipitation and evaporation rates that drive the hydrologic cycle as well as the winds regimes will also be affected (Roessig et al., 2004). Regarding the Ocean, similar trends are expected for sea surface temperature due to the warming of the surface mixed layer (Levitus et al., 2005) and the sea level will rise mainly because of the melting ice. Moreover, as the oceans are major carbon sink absorbing about 25% of anthropogenic CO_2, the sea surface CO_2 partial pressure (pCO_2) is also expected to increase (Sabine et al., 2004). This causes major shifts in seawater carbonate chemistry and is likely to reduce pH by 0.2-0.4 units, a phenomenon called “ocean acidification”, over the course of this century (Caldeira and Wickett, 2005).

In the northern Europe, the main scenario for climate change is milder, wetter and stormier winters (IPCC, 2007). Summers could be warmer and dryer, although the climatic effect is expected to be less pronounced than in winters (Jonsson and Jonsson, 2009). Simultaneously, the increasing precipitations will directly and indirectly impact the marine coastal area, causing a decrease of the salinity and amplifying the anthropogenic factors, such as hypoxic events linked to increased agricultural runoff or contamination of the nearshore zones by organic and inorganic contaminants released in the environment (Harley et al., 2006). Thus, the marine biota will have to deal with interactions between increasing seawater temperature, $p\mathrm{CO}_2$, and well-known anthropogenic stressors such as the contamination by heavy metals.

In ecotoxicology and risk assessment studies, it is widely accepted that the early life stages are the most sensitive to metallic contamination (*e.g.* Calabrese et al., 1973; Warnau et al., 1996; Oral et al., 2010). Recently, a growing body of works also showed that embryos and larvae were affected by hypercapnia (*e.g.* Kurihara et al., 2008; Dupont et al., 2008; Walther et al., 2010) as their developing systemic acid-base regulation is not yet fully efficient (Pörtner and Farrell, 2008). As the highly productive coastal zones serve as nursery grounds for many marine species of ecological and commercial importance (Dunn et al., 1999; Selleslagh and Amara, 2008), studying interaction between multiple stressors is critical to project the future of coastal ecosystems (Harley et al., 2006; Dupont et al., 2010).

In Europe, the common cuttlefish *Sepia officinalis* is seasonally exploited in the English Channel (Royer et al., 2006) when adults come in the shallow waters to mate and spawn their eggs before massive die-offs (Boucaud-Camou and Boismery, 1991). The eggs laid in shallow waters are subject to chronic exposure to various contaminants among which, heavy metals that bioaccumulate in the cuttlefish during its ontogenic development (Miramand et al., 2006; Villanueva and Bustamante, 2006). Experimental studies demonstrated that the cuttlefish embryo is partially protected because of the selective permeability of the eggshell to trace elements, depending on the developmental stage (Bustamante et al., 2002, 2004, 2006a; Lacoue-Labarthe et al., 2008a, 2009a, 2010). Moreover, these shielding properties of the egg capsule against Ag, Cd and Zn penetration and the metal accumulation efficiencies of the embryo seemed to be affected when eggs develop under warmer and hypercapnic conditions (Lacoue-Labarthe et al., 2009b).

This study addresses the combined effects of elevated temperature and pCO_2 on the bioaccumulation of selected radionuclides and trace elements in the cuttlefish embryo. Indeed, the development of nuclear facilities and fallout from nuclear weapon testing resulted in the release of several medium- and long-lived radionuclides into aquatic environments such as ^{241}Am, ^{60}Co, ^{134}Cs, ^{54}Mn or ^{75}Se (e.g. Warnau et al., 1999, Ke et al., 1999). Although these radionuclides are generally considered as micropollutants in the oceans, they are known to accumulate in marine organisms (e.g. Miramand and Guary, 1981; Bustamante et al. 2006b, Lacoue-Labarthe et al., 2010; Metian et al., 2009, 2011). In addition, radionuclides are of specific interest given the ecotoxicological concern regarding their stable isotopes. Indeed, Co, Mn and Se are trace elements that have essential functions in physiology but also toxic effect if they are excessively accumulated in organisms (*e.g.* Rainbow, 2002). In this study, we therefore use radiotracers to investigate the bioaccumulation behaviour of these corresponding stable elements that are present in marine waters (Warnau and Bustamante, 2007).

MATERIALS AND METHODS

Adult cuttlefish were collected by net-fishing off Monaco in April 2008 and maintained in open-circuit tanks in the IAEA-MEL premises. After mating, the fertilized eggs that were laid by each female were immediately separated to optimise their oxygenation. The eggs (n = 300) were placed and kept in six 5-L plastic bottles (50 eggs per bottle; one bottle per treatment; 0.45µm and UV-sterilized seawater; constantly aerated closed-circuit; salinity 38 p.s.u.; light/dark cycle: 12h/12h) during the full development time in controlled conditions of temperature and pH in a crossed (2 temperatures x 3 pH levels) experimental design. The pH and temperature values were chosen consistent with those of realistic modelled scenarios of climate change that would occur at the end of the century (Orr et al., 2005; Solomon et al., 2007). Temperature was controlled in each bath to within ± 0.5°C using temperature controllers connected to 300 W submersible heaters. The pH was controlled in each bottle to within ± 0.05 pH unit with a continuous pH-stat system (IKS, Karlsbad) that bubbled pure CO_2 into the bottles that were continuously aerated with CO_2-free air. The pH was maintained at a mean (± SD) of 7.61 ± 0.11, 7.84 ± 0.04, and 8.09 ± 0.04, at ambient temperature (16.0 ± 0.1°C), and of 7.61 ± 0.08, 7.84 ± 0.04, and 8.09 ± 0.09, at elevated temperature (18.9 ± 0.3°C), corresponding to pCO_2 of 1399, 781, and 404 ppm at ambient temperature and 1440, 799, and 399 ppm at elevated temperature, respectively (Lacoue-Labarthe et al., 2009b). The eggs were exposed to dissolved radiotracer: 0.27 kBq l^{-1} ^{241}Am, 0.95 kBq l^{-1} ^{60}Co, 1.18 kBq l^{-1} ^{134}Cs (Amersham, UK) and 0.91 kBq l^{-1} ^{54}Mn and 1.04 kBq l^{-1} ^{75}Se (Isotope Product Laboratory, USA). Briefly, seawater of each aquarium was spiked with typically 5 µl of radioactive stock solution (^{241}Am dissolved in 1N HCl, ^{60}Co in 0.1N HCl, ^{134}Cs in H_2O, ^{54}Mn in 0.1N HCl and ^{75}Se in 0.1N HCl). Seawater and spikes were renewed daily during the first week and then every second day to maintain good water quality and radiotracer concentrations as constant as possible. The detailed experimental procedure has been previously described in Lacoue-Labarthe et al. (2009b).

At hatching time (i.e., 62 d and 42 d at 16 and 19°C, respectively, when first hatching events occurred), in each treatment, 10 eggs were fresh weighed and dissected and the radiotracer activities were counted in the eggshell, the embryo and the perivitelline fluid (PVF). In parallel, 10 newly hatched cuttlefish in each bottle were weighted and counted.

Radioactivities were measured using a high-resolution γ-spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyzer and a computer equipped with a spectra analysis software (Interwinner® 6). Radioactivities were determined by comparison with standards of known activity and of appropriate geometry. Measurements were corrected for counting efficiency and physical radioactive decay. Counting times were adapted to obtain relative propagated errors less than 5% (Metian et al., 2008).

Accumulation of radiotracers was expressed as concentration factors (CF), which is the ratio between radiotracer activity in the egg or egg compartment (Bq g^{-1}) and the time-integrated activity in seawater (Bq g^{-1}) (Metian et al., 2008). The distribution of metals was expressed a % of the total activity load. Results are expressed as mean ± SD.

RESULTS

At hatching time, only ^{60}Co, ^{134}Cs and ^{54}Mn were accumulated with sufficient activities to be detected in the newly hatched juveniles, showing that ^{241}Am and ^{75}Se were not efficiently accumulated during the embryonic development. The lower the pH of seawater where eggs were incubating, the less ^{60}Co and ^{54}Mn were accumulated in the tissues of hatchlings (Fig. 1; Table 1). Moreover, this effect tended to be reduced when the temperature increases, i.e. ^{60}Co and ^{54}Mn CF values were 1.5 and 1.1 fold lower at pH 7.60 than at normal pH, when eggs developed at 16 and 19°C, respectively. Finally, neither the temperature nor the pH has an effect on the ^{134}Cs CF values in the hatchlings.

At the end of the embryonic development, radiotracer distribution was determined among the different eggs compartments (Table 2). Regardless the temperature and pCO_2, over 80% of both radionuclides and trace elements were found associated with the eggshell, with the exception of ^{134}Cs that was found in variable proportions in the eggshell, the embryo and the PVF. More precisely, the fraction of Cs in PVF was higher at lower pH levels than at normal pH (KW test; $p < 0.05$), whereas the one in the eggshell was lower at pH_T 7.60 than at pH_T 8.10 (KW test; $p < 0.05$). No effect of the temperature was noted on the Cs fraction in PVF (KW test; $p > 0.05$). Concerning Se, this element was slightly detected in the PVF but not in the embryo.

DISCUSSION

Studying the impact of the ocean acidification on the metal accumulation on water-breathing animals must raise attention on two aspects. On the one hand, the speciation of some metals and their bioavailability could be affected as ^{241}Am that form strong complexes with hydroxide and carbonate ions (Choppin, 2006), both of which being expected to decrease in seawater (Feely, 2004). Other metals mainly found as free forms (Co^{2+}, Cs^{+}, Mn^{2+}), or oxydized state (SeO_4^{2-}) will be more available by only few percent with decreasing pH (Byrne, 2002). Moreover, the increase in the concentration of H^{+} could reduce by competition for the binding site the metal adsorption on the eggshell surface or epithelia (Millero et al., 2009). On the other hand, the acid-base balance and ionoregulation impairments (Pörtner, 2008) caused by hypercapnia could modify the active transport or passive diffusion of metals through the epithelial membrane (Rainbow, 1997) and therefore the accumulation efficiencies in the animal tissues.

As noticed in previous studies for different elements (Bustamante et al., 2002, 2004, 2006a; Lacoue-Labarthe et al., 2008a, 2009a, 2010) the behaviour of trace elements towards cuttlefish egg strongly varies according to the considered elements. Among the three element found in the embryo, Co and Mn accumulation decreased with pH as previously observed with Cd (Lacoue-Labarthe et al., 2009b). It is likely that both these “Class Borderline” elements (Nieboer and Richardson, 1980) might be subjected to increased competition with protons for the adsorption on binding sites on embryonic tissues. Nevertheless, regarding the direct effect of temperature with similar CF at 19°C than at 16°C despite of a shorter exposure duration, we assume that the presumably increased metabolic level of the embryo could contribute to these essential element uptake efficiencies (Rainbow and White, 1990; Barceloux, 1999). The seawater hypercapnia would in this case enhance the metabolic depression of the late-stage embryo (Rosa and Seibel, 2008; Melzner et al., sbm) and limit active metal accumulation. Contrasting to Co and Mn, Se passed slightly through the eggshell but was not significantly accumulated by the embryo, suggesting that the need of this essential metal (Bell et al., 1986) was entirely supplied by the maternal transfer through Se incorporation in the yolk reserve (Lacoue-Labarthe et al., 2008b). More globally, these results raise the question of the impact of a potential depletion in essential elements during the sensitive early-life stages of this marine invertebrate.

Regarding the radionuclide behaviours, the absence of ^{241}Am neither in the embryo nor in the PVF confirms that the eggshell, retaining more than 98% of the Am amount, totally prevents against ^{241}Am penetration (Bustamante et al., 2006a; Lacoue-Labarthe et al., 2010) and that pH and temperature variations did not affect these shielding properties although the proportion of bioavailable Am increase with decreasing pH (Choppin, 2006). Oppositely, the eggshell displayed an evident permeability toward ^{134}Cs (Bustamante et al., 2006a) as CF values were > 10 in the embryo. Interestingly, decreasing pH and increasing temperature did not affect Cs uptake in the embryo but enhanced the proportion associated with the PVF compartment (Table 2). This probably results from the enhanced egg swelling illustrated by higher egg weight under hypercapnic conditions and warm water (Lacoue-Labarthe et al., 2009b). Therefore the fraction of ^{134}Cs, already described as a tracer of water movement during the cuttlefish egg development (Lacoue-Labarthe et al., 2010), increased with the peri-vitelline volume.

In conclusion, this study demonstrates that both temperature and pH have distinct effects on the bioaccumulation in cuttlefish embryos. Both the chemical properties of the elements and the physiological responses of the organism to ocean warming and acidification could account for the observed effects. The impacts of pollutants in the context of the global change, such as the potential depletion of essential elements in embryo, should to be further assessed in marine organisms that use coastal areas to complete their life cycle.

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Table 1. *Sepia officinalis*. Two-way ANOVA parameters testing the effects of three pH (7.60, 7.85 and 8.10) and two temperatures (16 and 19°C) on the weight of the eggs (from Lacoue-Labarthe et al [33]), and on the concentration factor (CF) of ^{60}Co, ^{134}Cs and ^{54}Mn in the hatchlings at the end of the embryonic development (see Figures 1).

Parameter	pH			Temperature			pH X Temperature		
	df	MS	F	*df*	MS	F	*df*	MS	F
Egg weight	2	0.587	8.8^{***}	1	1.486	22.2^{***}	2	0.018	0.3^{ns}
^{60}Co CF	2	20424	17.3^{***}	1	454	0.7^{ns}	2	7465	6.3^{**}
^{134}Cs CF	2	168	0.9^{ns}	1	345	1.8^{ns}	2	138	0.7^{ns}
^{54}Mn CF	2	2152	17.3^{***}	1	1316	10.6^{**}	2	2230	18.0^{***}

df = degree of freedom; MS = mean squares. Probability levels for significant effects: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p < 0.1$ (·); ns = non significant.

Table 2. *Sepia officinalis*. Distribution ^{241}Am, ^{60}Co, ^{134}Cs, ^{54}Mn and ^{75}Se expressed as % among the different egg compartments, at the end of development (after 62 d and 42 d of incubation at 16°C and 19°C, respectively) following three different pH levels at two different temperatures.

Experiment	16°C			19°C		
	7.6	7.85	8.1	7.6	7.85	8.1
(a) ^{241}Am						
Egshell	98.8 ± 0.2	98.8 ± 0.7	98.2 ± 1.2	96.9 ± 2.2	98.8 ± 0.8	98.3 ± 1.0
Embryo	< 1	< 1	< 1	< 1	< 1	< 1
PVF	< 1	< 1	< 1	< 1	< 1	< 1
(b) ^{60}Co						
Eggshell	88.4 ± 0.9	87.2 ± 2.1	90.5 ± 2.6	83.1 ± 1.8	89.7 ± 1.0	91.4 ± 0.4
Embryo	10.2 ± 0.8	11.5 ± 2.1	8.5 ± 2.6	15.0 ± 1.3	9.0 ± 0.9	8.4 ± 0.5
PVF	1.4 ± 0.1	1.3 ± 0.2	0.9 ± 0.2	1.9 ± 0.5	1.3 ± 0.4	0.2 ± 0.1
(c) ^{134}Cs						
Eggshell	27.8 ± 11.7	33.0 ± 8.0	52.7 ± 17.6	19.2 ± 3.1	24.9 ± 14.1	48.6 ± 19.5
Embryo	30.6 ± 14.9	34.0 ± 7.1	30.3 ± 14.6	39.4 ± 5.0	36.0 ± 14.3	42.5 ± 18.1
PVF	41.6 ± 8.9	33.0 ± 1.7	17.0 ± 4.9	41.3 ± 5.7	39.1 ± 6.1	8.9 ± 2.1
(d) ^{54}Mn						
Eggshell	88.9 ± 1.3	88.8 ± 0.3	88.0 ± 2.6	84.8 ± 2.1	90.2 ± 1.7	83.4 ± 1.9
Embryo	8.7 ± 0.8	8.9 ± 0.5	10.0 ± 3.0	12.8 ± 2.0	8.1 ± 1.3	15.5 ± 1.7
PVF	2.4 ± 1.0	2.3 ± 0.4	2.0 ± 0.7	2.4 ± 0.5	1.7 ± 0.4	1.1 ± 0.4
(e) ^{75}Se						
Eggshell	97.2 ± 0.4	97.5 ± 0.4	98.3 ± 0.3	95.0 ± 1.3	96.1 ± 0.4	98.0 ± 0.6
Embryo	< 1	< 1	< 1	< 1	< 1	< 1
PVF	2.0 ± 0.2	2.0 ± 0.5	1.1 ± 0.3	3.6 ± 0.8	2.7 ± 0.3	1.0 ± 0.4

PVF: perivitelline fluid. Letters denote statistically significant differences (Kruskall-Wallis test; $p < 0.05$) between pHs for each temperature.

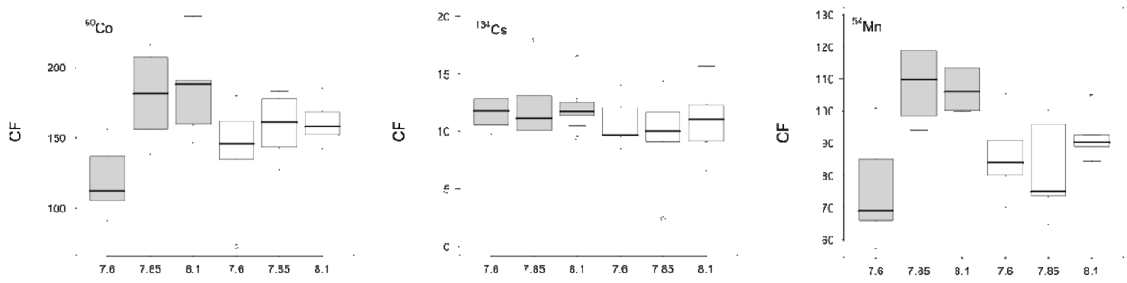

Figure 1. *Sepia officinalis.* Concentration factors of ^{60}Co, ^{134}Cs and ^{54}Mn (CF; n = 10), in the newly hatched juvenile exposed at three different pH at 16°C (grey) and 19°C (white) during the whole development time, *i.e.* 62 d and 42 d, respectively. ^{241}Am and ^{75}Se activities levels were under detection limits. Results of the statistical analysis were reported on the Table 1.